AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

PUBLIC AND ENVIRONMENTAL HEALTH MICROBIOLOGY

Local and Transboundary Transmissions of Methicillin-Resistant *Staphylococcus aureus* Sequence Type 398 through Pig Trading

Mattia Pirolo,[a] Raphael N. Sieber,[b] Arshnee Moodley,[c,d] Daniela Visaggio,[a] Irene Artuso,[a] Angela Gioffrè,[e] Francesco Casalinuovo,[f] Giovanna Spatari,[g] Luca Guardabassi,[c,h] Marc Stegger,[b] Paolo Visca[a]

[a]Department of Science, Roma Tre University, Rome, Italy
[b]Department of Bacteria, Parasites and Fungi, Statens Serum Institut, Copenhagen, Denmark
[c]Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark
[d]CGIAR AMR Hub, International Livestock Research Institute, Nairobi, Kenya
[e]Department of Medicine, Epidemiology, Workplace and Environmental Hygiene, Lamezia Terme Research Centre, INAIL–National Institute for Insurance against Accidents at Work, Lamezia Terme, Italy
[f]Istituto Zooprofilattico Sperimentale del Mezzogiorno, Catanzaro, Italy
[g]Department of Biomedical Sciences, Dental, Morphological and Functional Investigations, University of Messina, Messina, Italy
[h]Department of Pathobiology and Population Sciences, Royal Veterinary College, Hatfield, United Kingdom

Mattia Pirolo, Raphael N. Sieber, Marc Stegger, and Paolo Visca contributed equally to this work. Author order was determined both alphabetically and in order of increasing seniority.

ABSTRACT Livestock-associated methicillin-resistant *Staphylococcus aureus* sequence type (ST) 398 (LA-MRSA ST398) is a genetic lineage for which pigs are regarded as the main reservoir. An increasing prevalence of LA-MRSA ST398 has been reported in areas with high livestock density throughout Europe. In this study, we investigated the drivers contributing to the introduction and spread of LA-MRSA ST398 through the pig farming system in southern Italy. Whole-genome sequencing (WGS) of LA-MRSA ST398 isolates collected in 2018 from pigs ($n = 53$) and employees ($n = 14$) from 10 farms in the Calabria region of Italy were comparatively analyzed with previously published WGS data from Italian ST398 isolates ($n = 45$), an international ST398 reference collection ($n = 89$), and isolates from Danish pig farms ($n = 283$), which are the main suppliers of pigs imported to Italy. Single-nucleotide polymorphisms (SNP) were used to infer isolate relatedness, and these data were used together with data from animal trading to identify factors contributing to LA-MRSA ST398 dissemination. The analyses support the existence of two concurrent pathways for the spread of LA-MRSA ST398 in southern Italy: (i) multiple introductions of LA-MRSA ST398 through the import of colonized pigs from other European countries, including Denmark and France, and (ii) the spread of distinct clones dependent on local trading of pigs between farms. Phylogenetically related Italian and Danish LA-MRSA ST398 isolates shared extensive similarities, including carriage of antimicrobial resistance genes. Our findings highlight the potential risk of transboundary transmission of antimicrobial-resistant bacterial clones with a high zoonotic potential during import of pigs from countries with high LA-MRSA prevalence.

IMPORTANCE Over the past decade, livestock-associated methicillin-resistant *Staphylococcus aureus* sequence type 398 (LA-MRSA ST398) has spread among pig holdings throughout Europe, in parallel with the increased incidence of infections among humans, especially in intensive pig farming regions. Despite the growing prevalence of LA-MRSA ST398 in Italian pig farms, the transmission dynamics of this clone in Italy remains unclear. This work provides genome-based evidence to suggest transboundary LA-MRSA ST398 transmission through trading of colonized pigs between Euro-

Citation Pirolo M, Sieber RN, Moodley A, Visaggio D, Artuso I, Gioffrè A, Casalinuovo F, Spatari G, Guardabassi L, Stegger M, Visca P. 2020. Local and transboundary transmissions of methicillin-resistant *Staphylococcus aureus* sequence type 398 through pig trading. Appl Environ Microbiol 86:e00430-20. https://doi.org/10.1128/AEM.00430-20.

Editor Charles M. Dozois, INRS—Institut Armand-Frappier

Address correspondence to Paolo Visca, paolo.visca@uniroma3.it.

Received 21 February 2020
Accepted 22 April 2020
Accepted manuscript posted online 1 May 2020
Published 17 June 2020

pean countries and Italy, as well as between farms in the same Italian region. Our findings show that both international trading and local trading of colonized pigs are important factors contributing to the global spread of LA-MRSA ST398 and underscore the need for control measures on and off the farm to reduce the dissemination of this zoonotic pathogen.

KEYWORDS animal movement, livestock, MRSA, porcine, ST398, whole-genome sequencing, zoonosis

Staphylococcus aureus is an opportunistic human pathogen that can cause a variety of diseases, ranging from skin and soft tissue infections to life-threatening invasive infections. Some of these infections are caused by drug-resistant strains, primarily methicillin-resistant *S. aureus* (MRSA). Since the mid-2000s, MRSA clones colonizing livestock animals, called livestock-associated MRSA (LA-MRSA) (1), have emerged. The most common LA-MRSA lineage in the European Union (EU) is sequence type 398 (ST398).

Since the first EU baseline survey in 2008 (2), an increase in the prevalence of LA-MRSA ST398 has been documented in several EU countries (3–6). Worryingly, this lineage has spread beyond the farm setting, showing increasing prevalence among humans living in high-density livestock production areas (7, 8).

The application of high-throughput whole-genome sequencing (WGS) has unveiled potential drivers for LA-MRSA ST398 dissemination, and trading of colonized pigs, contaminated transport vehicles, and human carriers have been suggested as potential vectors for both local and transboundary transmission of LA-MRSA (4, 5, 9).

Italy is the sixth largest pork producer in the EU (10), and in 2008, two nationwide surveys estimated the prevalence of LA-MRSA ST398 among pig farms in Italy to be 14% to 28% (2, 11). Since then, the prevalence of LA-MRSA ST398 in the Italian pig farming system has steadily increased, especially in southern Italy, where the percentage of positive farms has been estimated to be ca. 60% (6, 12). However, the transmission routes that have caused such a major increase of LA-MRSA ST398 prevalence have not been investigated so far.

In this study, we integrated WGS data for LA-MRSA ST398 isolated from pigs farmed in southern Italy (6, 13) with genome data available in international sequence repositories in order to trace the local and transboundary dissemination dynamics of LA-MRSA ST398.

RESULTS

Selection of LA-MRSA ST398 isolates for WGS. WGS was performed on 67 recently isolated LA-MRSA ST398 strains representative of the major clones, as defined by *spa* typing, circulating among pigs and farm workers in a large area of southern Italy (Calabria region; 15,222 km^2). Fifty-three strains originated from pigs and 14 from farm workers. All of them were isolated in intensive farms during a survey conducted in 2018 (6, 13). Antibiotic resistance profiles and *spa* and SCC*mec* types were previously determined (see Table S1 in the supplemental material). Isolates were selected according to the criteria described in Materials and Methods.

Phylogeographic context and comparative genomics of LA-MRSA ST398. Animal movement is considered a driver for LA-MRSA ST398 spread among pig farms (5, 9, 14). To trace the source of recently identified Italian LA-MRSA ST398 isolates to a potential country of origin, the genomes of the 67 LA-MRSA ST398 isolates from southern Italy were compared with 45 genomes from previously sequenced Italian *spa* type t899-related ST398 isolates (15) and an international reference collection of 89 genomes of methicillin-resistant and -susceptible *S. aureus* ST398 (16) strains, including the *S. aureus* ST398 reference strain S0385. In total, 201 genomes were investigated to reconstruct phylogenetic relationships based on single-nucleotide polymorphisms (SNPs).

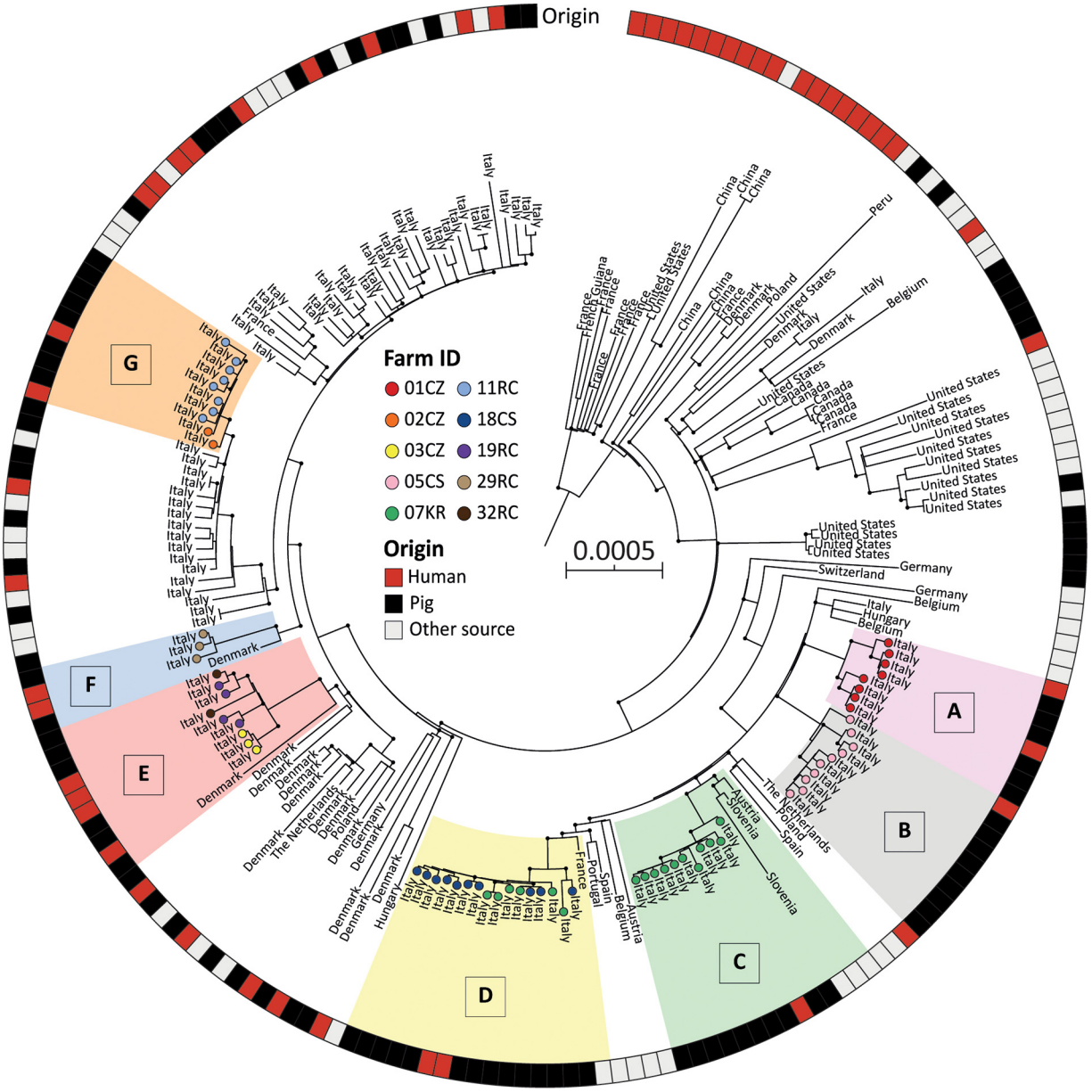

FIG 1 Rooted maximum-likelihood phylogeny of 67 recent LA-MRSA ST398 isolates from southern Italy, 89 *S. aureus* ST398 isolates from the international reference collection (16), and 45 Italian LA-MRSA ST398 t899-related isolates (15). The tree was rooted according to the outgroup used in reference 16. Bootstrap values above 90% are illustrated by filled circles at the ends of branches. Colored filled circles correspond to farms yielding isolates. The scale bar represents the number of nucleotide substitutions per variable site. A to G represent groups of the recent Italian isolates and their closest neighbors from the international reference collection.

After removal of 327 sites in recombinant regions, 6,400 core genome SNPs in the 201 isolates were used to construct a rooted maximum-likelihood tree (Fig. 1; also, see Fig. S1 in the supplemental material). The analysis revealed a nonuniform distribution of the isolates from southern Italy, which appeared intermingled throughout the phylogeny and did not cluster according to geographic origin (Fig. 1). Seven groups (designated A to G) (Fig. 1) comprising the recent isolates from southern Italy with their closest neighbors from the international reference collection (16) were arbitrarily defined. All groups were supported with bootstrap values of >90%. Groups A, B, C, and F were composed solely of isolates originating from single farms (designated 01CZ, 05CS, 07KR, and 29RC, respectively). Six isolates from farm 07KR clustered with isolates from farm 18CS (group D). Groups E and G were composed of isolates originating from three farms (03CZ, 19RC, and 32RC) and two farms (02CZ and 11RC), respectively.

TABLE 1 Association between source of pigs and distribution of LA-MRSA ST398 isolates across the phylogeny

Farm	Source of swine	Isolate cluster or lineage
01CZ	Spain	Unique Italian cluster
02CZ	Northern Italy	Italian t899 lineage
03CZ	Denmark	Danish lineage L3[a]
05CS	None[b]	Unique Italian cluster
07KR	France	Danish lineage L2[a] (11/17 isolates) and unique Italian cluster (6/17 isolates) related to a French isolate
11RC	Northern Italy	Italian t899 lineage
18CS	Southern Italy	Pigs purchased from farm 07KR; isolates formed a unique Italian cluster with isolates from farm 07KR
19RC	Denmark	Danish lineage L3[a]
29RC	Denmark	Danish lineage L1[a]
32RC	France	Danish lineage L3[a]

[a]According to reference 5.
[b]Farm with a closed-cycle breeding system.

The relatedness of isolates from southern Italy and other countries was examined using the phylogeny (Fig. 1). Groups A, B, and G did not exhibit any close relationship with isolates from countries other than Italy. Conversely, the closest neighbors of Italian isolates clustering in groups C, D, E, and F originated from different EU countries (16). Group C was related to isolates from Austria and Slovenia, whereas group D was related to one French isolate. Both groups E and F were closely related to isolates from Denmark.

Evidence of transboundary and local transmission of LA-MRSA ST398. To investigate any possible transboundary and/or local dissemination of LA-MRSA ST398 via pig movement, farms from which the 67 isolates originated from were queried about the source of their pigs (Table 1). Three farms (03CZ, 19RC, and 29RC) reported that they had purchased animals from Denmark (Table 1), which in 2015 was the country with the highest number of pigs imported into Italy (17) (Fig. 2) and which has experienced a remarkable increase in LA-MRSA ST398 prevalence in pig farming (18). Since the structure of the LA-MRSA ST398 population in Danish pigs was recently characterized by WGS analysis (5), Denmark was selected as the study case to uncover potential LA-MRSA ST398 transmission through pig trading to southern Italy. To this purpose, additional genome data for isolates from Danish pig farms ($n = 283$) (5) were incorporated in the analyses.

A total of 484 isolates were included in this analysis, and they differed in 6,059 core genome SNPs, after the removal of 591 sites falling into recombination regions. The

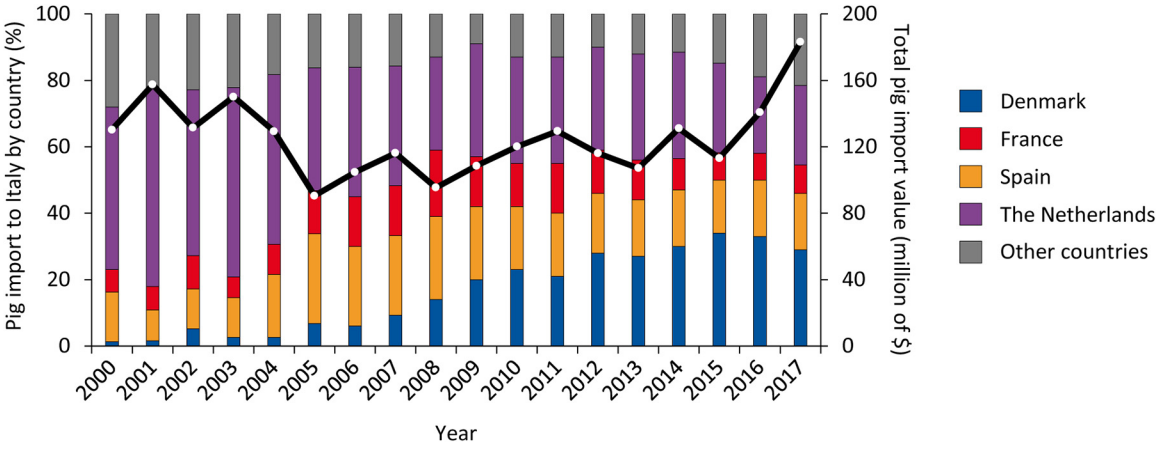

FIG 2 Data on import of pigs to Italy by country of origin and year. White dots denote the total import value of pigs per year, expressed in millions of U.S. dollars, with the solid black line showing the yearly trend. Data were retrieved from the Observatory of Economic Complexity (17).

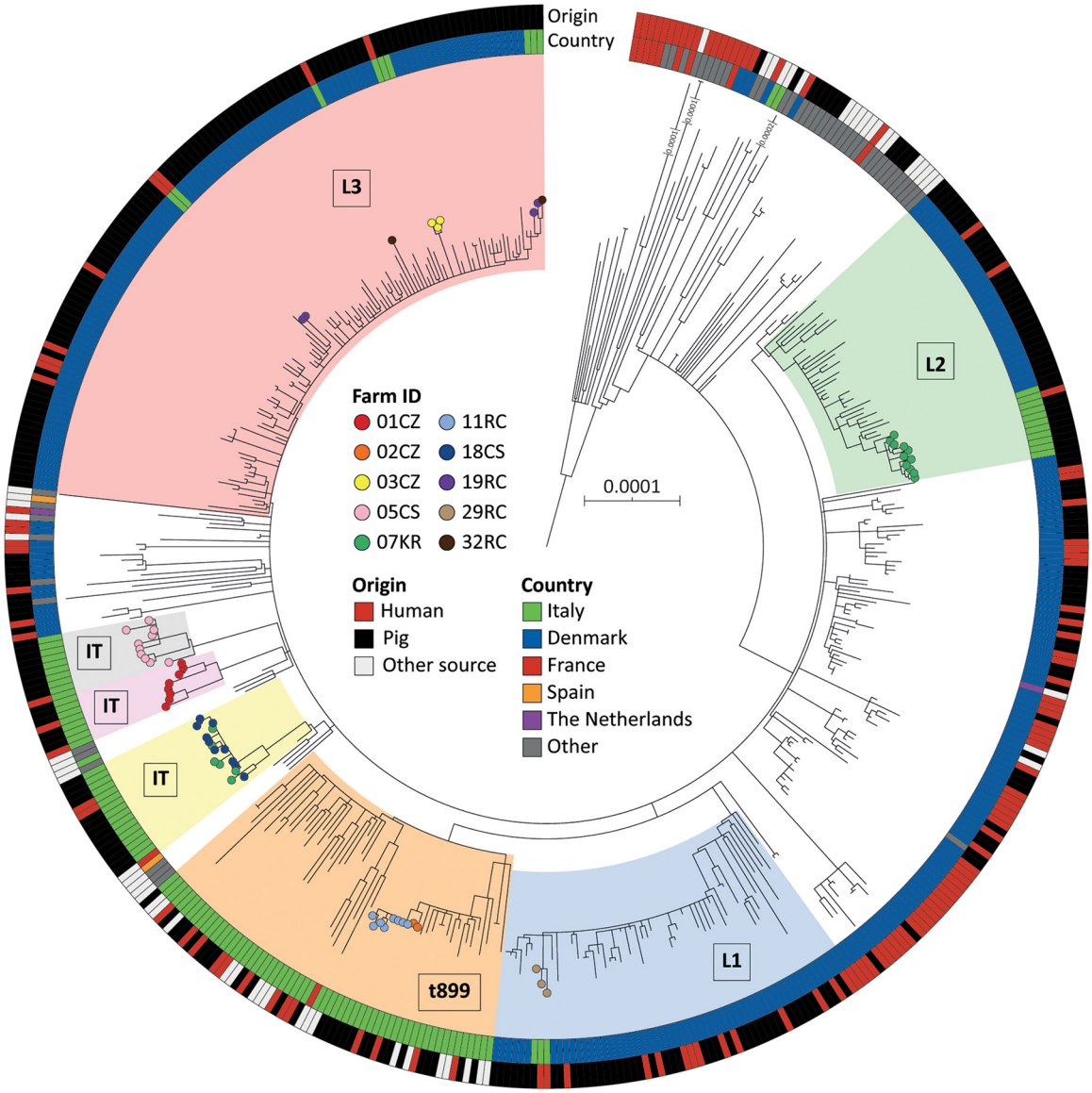

FIG 3 Rooted maximum-likelihood phylogeny of 67 recent LA-MRSA ST398 isolates from southern Italy, 283 LA-MRSA ST398 Danish isolates (5), 89 *S. aureus* ST398 isolates from the international reference collection (16), and 45 Italian LA-MRSA ST398 t899-related isolates (15) (see Fig. S2 in the supplemental material for further annotations, including bootstrap values). The tree was rooted according to the outgroup used in reference 16. Colored filled circles correspond to farms yielding isolates. The scale bar represents the number of nucleotide substitutions per variable site. IT, Italian cluster; L1, Danish lineage 1; L2, Danish lineage 2; L3, Danish lineage 3; t899, Italian t899 lineage.

rooted SNP-based maximum-likelihood tree is shown in Fig. 3 (see Fig. S2 for additional details). The isolates from the three Italian farms importing animals from Denmark (farms 03CZ, 19RC, and 29RC) (Table 1) clustered with the prevalent L1 and L3 Danish lineages (5) (Fig. 3). Two farms (07KR and 32RC) reported importing pigs from France, despite the fact that their isolates exhibited a close relatedness with the Danish L2 and L3 lineages.

Three unique Italian clusters are defined in Fig. 3; two of them are composed of isolates from farms 01CZ and 05CS (corresponding to groups A and B in Fig. 1, respectively). Farm 01CZ imported animals from Spain, while farm 05CS had an autonomous breeding system (Table 1). The third Italian cluster (corresponding to group D in Fig. 1) was composed of isolates from farms reporting interfarm trading of pigs (farm 07KR sold pigs to farm 18CS [6]) (Table 1). Interestingly, farm 07KR imported

TABLE 2 Antimicrobial resistance genes in Italian and Danish isolates belonging to lineages L1, L2, and L3[a]

Antimicrobial and resistance gene	No. of positive isolates (%)								
		L1		L2		L3		L1 + L2 + L3	
	IT ($n = 67$)	IT ($n = 3$)	DK ($n = 54$)	IT ($n = 11$)	DK ($n = 34$)	IT ($n = 9$)	DK ($n = 105$)	IT ($n = 23$)	DK ($n = 193$)
Aminoglycoside	55 (82.1)	3 (100)	47 (87.0)	11 (100)	16 (47.1)**	9 (100)	57 (54.3)**	23 (100)	120 (62.2)***
aac(6′)-aph(2″)	12 (17.9)	0 (0)	0 (0)	0 (0)	1 (2.9)	0 (0)	0 (0)	0 (0)	1 (0.5)
aadD	14 (20.9)	3 (100)	41 (75.9)	0 (0)	4 (11.8)	0 (0)	2 (1.9)	3 (13.0)	47 (24.4)
aadE	3 (4.5)	3 (100)	42 (77.8)	0 (0)	0 (0)	0 (0)	0 (0)	3 (13.0)	42 (21.8)
ant(9)-Ia	34 (50.7)	0 (0)	0 (0)	11 (100)	1 (2.9)	9 (100)	2 (1.9)***	20 (87.0)	3 (1.6)***
aph(6)-Ic	3 (4.5)	0 (0)	7 (13.0)	0 (0)	14 (41.2)**	3 (33.3)	55 (52.4)	3 (13.0)	76 (39.4)*
Beta-lactam	67 (100)	3 (100)	54 (100)	11 (100)	34 (100)	9 (100)	105 (100)	23 (100)	193 (100)
mecA	67 (100)	3 (100)	54 (100)	11 (100)	34 (100)	9 (100)	105 (100)	23 (100)	193 (100)
blaZ	53 (79.1)	1 (33.3)	52 (96.3)*	8 (72.7)	1 (2.9)***	9 (100)	105 (100)	18 (78.3)	158 (81.9)
Cadmium/zinc	57 (85.1)	3 (100)	46 (85.2)	11 (100)	34 (100)	9 (100)	101 (96.2)	23 (100)	181 (93.8)
czrC	57 (85.1)	3 (100)	46 (85.2)	11 (100)	34 (100)	9 (100)	101 (96.2)	23 (100)	181 (93.8)
Lincosamide	42 (62.7)	3 (100)	43 (79.7)	11 (100)	28 (82.4)	9 (100)	105 (100)	23 (100)	176 (91.2)
lnu(A)	5 (7.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
lnu(B)	37 (55.2)	3 (100)	43 (79.7)	11 (100)	28 (82.4)	9 (100)	105 (100)	23 (100)	176 (91.2)
Macrolide	25 (37.3)	0 (0)	22 (40.7)	11 (100)	24 (70.6)	3 (33.3)	21 (20.0)	14 (60.9)	67 (34.7)*
erm(B)	12 (17.9)	0 (0)	0 (0)	11 (100)	23 (67.6)*	0 (0)	5 (4.8)	11 (47.8)	28 (14.5)***
erm(C)	13 (19.4)	0 (0)	22 (40.7)	0 (0)	4 (11.8)	3 (33.3)	17 (16.2)	3 (13.0)	43 (22.3)
Phenicol	10 (14.9)	0 (0)	1 (1.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)
fexA	10 (14.9)	0 (0)	1 (1.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)
Streptogramin B	10 (14.9)	0 (0)	1 (1.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)
vga(A)	10 (14.9)	0 (0)	1 (1.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)
Tetracycline	67 (100)	3 (100)	54 (100)	11 (100)	34 (100)	9 (100)	105 (100)	23 (100)	193 (100)
tet(K)	50 (74.6)	1 (33.3)	48 (88.9)	10 (90.9)	34 (100)	9 (100)	100 (95.2)	20 (87.0)	182 (94.3)
tet(L)	11 (16.4)	0 (0)	0 (0)	0 (0)	6 (17.6)	0 (0)	1 (1.0)	0 (0)	7 (3.6)
tet(M)	67 (100)	3 (100)	54 (100)	11 (100)	34 (100)	9 (100)	102 (97.1)	23 (100)	190 (98.4)
Trimethoprim	28 (41.8)	3 (100)	54 (100)	7 (63.6)	13 (38.2)	9 (100)	105 (100)	19 (82.6)	172 (89.1)
dfrG	23 (34.3)	3 (100)	54 (100)	7 (63.6)	7 (20.6)*	9 (100)	105 (100)	19 (82.6)	166 (86.0)
dfrK	5 (7.5)	0 (0)	0 (0)	0 (0)	6 (17.6)	0 (0)	1 (1.0)	0 (0)	7 (3.6)

[a]DK, Danish isolates (5); IT, Italian isolates (this study); L1, L2, and L3, Danish lineages 1, 2, and 3. Asterisks denote significant differences in the frequency of antimicrobial genes between Italian and Danish isolates of the same lineage: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

animals from France (Table 1), and the isolate from the international reference collection most closely related to this cluster also originated from France (Fig. 3). Lastly, all the t899-related isolates clustered together (t899 cluster) (Fig. 3), supporting the notion that *spa* type t899 represents a monophyletic entity (15, 16). Within this cluster, all isolates from southern Italy appeared to be closely related (group G in Fig. 1), originating from two farms importing animals from northern Italy (farms 02CZ and 11RC) (Table 1).

Distribution of antimicrobial resistance genes. The distribution of antimicrobial resistance genes among the 67 LA-MRSA ST398 isolates from southern Italy is reported in Table 2 and Fig. S3. The previously documented presence of *mecA* and *tet*(M) (6, 13) was confirmed in all isolates. Aminoglycoside resistance genes were present in >80% of the isolates, with *ant(9)-Ia* (synonymous with *spc*) being the most prevalent, followed by *aadD* and *aac(6′)-aph(2′′)* (Table 2). Interestingly, the zinc-cadmium resistance gene *czrC* was exclusively detected in isolates carrying SCC*mec* type V (85% of the isolates) (Fig. S3). The *erm*(B) and *erm*(C) genes, conferring resistance to macrolide-lincosamide-streptogramin B (MLS_B), were present in 18% and 19% of the isolates, respectively. Lincosamide resistance was encoded by *lnu*(B) and *lnu*(A) in 55.2% and 7.5% of the isolates, respectively. Similarly, trimethoprim resistance was encoded by *dfrG* and *dfrK* in 34.3% and 7.5% of the isolates, respectively.

The expansion of the L1, L2, and L3 lineages among Danish LA-MRSA ST398 isolates was suggested to be driven by the use of antimicrobials (5). To investigate the transmission of antimicrobial resistance genes along with pig movement, the antibiotic resistomes in Italian and Danish isolates of lineages L1, L2, and L3 were compared (Table 2). Italian isolates clustering within L1, L2, and L3 lineages showed resistance gene patterns very similar to those of the Danish isolates from the same lineages. When data for all three lineages were combined, significant differences between Italian and Danish isolates were observed for only three genes; specifically, *ant(9)-Ia* (spectinomycin resistance) was significantly more frequent among Italian isolates, whereas *aph(6)-Ic* (synonymous with *str*; streptomycin resistance) and *erm*(B) (macrolide resistance) were more frequent among Danish isolates (Table 2).

DISCUSSION

WGS of recent LA-MRSA ST398 isolates from southern Italy was performed to gain insights into the transmission dynamics within and between countries. Our findings strongly suggest the existence of two concurrent modes for LA-MRSA ST398 introduction and spread in southern Italy. The first is multiple introductions of LA-MRSA ST398 strains by trading of piglets with other EU countries, including France and Denmark. The second is the expansion of independent ST398 clones, especially in farms trading animals with other Italian farms.

With 13 million exported animals per year, Denmark is the leader in pig exports to other EU countries (19, 20), and our analysis revealed that Denmark has been the main provider of pigs to Italy since 2015. Denmark has experienced a dramatic increase in the prevalence of LA-MRSA ST398 in pig farms, and in 2018, >80% (83% to 89%) of the conventional pig farms were found to be positive for MRSA (18). This increase has been linked to the clonal expansion of three dominant lineages (L1, L2, and L3) (5), which have spread beyond the farm level and have been detected in the Danish food production chain and health care facilities (8, 21, 22). Since Denmark is the primary country of origin of pigs imported to Italy, genomic comparison of our isolates to Danish isolates revealed that isolates from Italian farms that reported pig imports from Denmark clustered within the dominant Danish lineages L1 and L3. This supports transmission of Danish LA-MRSA ST398 with pigs traded to Italy. Interestingly, farms purchasing animals from France also appeared to be colonized by strains belonging to the Danish lineages L2 and L3. It could be hypothesized that Danish lineages L2 and L3 previously spread to France and from there they were imported to Italy. However, only a few French strains were available in the ST398 reference data set (16), and they were scattered across the phylogeny. Thus, a larger and more recent collection of French isolates, as well as other national collections of LA-MRSA ST398 isolates from Europe, should be inspected to confirm our hypothesis.

Interestingly, Italian and Danish isolates clustering within the predominant Danish lineages (L1, L2, and L3) also shared a high similarity in carriage of antibiotic resistance genes which confer resistance to antimicrobials commonly used in the Danish pig farming system (18). The different frequency of *ant(9)-Ia* (spectinomycin) and *aph(6)-Ic* (streptomycin) gene carriage between Italian and Danish isolates could reflect the different use of aminoglycosides in the two countries. Moreover, recent isolates from southern Italy showed higher frequency of the zinc/cadmium resistance gene *czrC* (85%) than previously reported (56%) (23). This is consistent with the predominant SCC*mec* type V found among the Italian isolates and the extensive use of zinc oxide to prevent postweaning diarrhea in pigs (5, 24), which is still common practice in Italy.

This study has some limitations. First, despite the evident genetic relatedness between Danish and Italian MRSA isolates, the skewness of the Danish data set may have caused a bias toward the suggested geographical origin of MRSA in Italian pigs. Second, unique isolates from only 10 farms in a large region with high density of pig farming were analyzed, and the number of isolates per farm was not uniform (2 to 17 isolates). Third, ST398 has been shown to spread through the environment (e.g., via water outflow and manure or dust effusion), via contaminated fomites, and from

other hubs of dissemination which we cannot exclude. For example, farm 19RC not only imported colonized pigs from Denmark but also employed a colonized farmer (19RC002U) who regularly visited pig farms in Denmark. In this case, the possibility of travel-associated human colonization and/or human-to-pig transmission cannot be ruled out. However, no such links were documented in the other farms. Thus, our observations strongly suggest that local and transboundary transmission of multidrug-resistant ST398 occurred via trading of colonized animals, as already reported in Denmark, Norway, and New Zealand (4, 5, 9).

In summary, this study sets the groundwork for future WGS-based epidemiological investigations of LA-MRSA ST398 in Italy. The dissemination of this lineage is known to be facilitated by animal movements, and trading of ST398-positive pigs between Italian pig farms and holdings from other EU countries may have contributed to the spread of this lineage. Our findings underscore the need for control measures on and off the farm to reduce the dissemination of this zoonotic pathogen.

MATERIALS AND METHODS

Selection criteria of LA-MRSA ST398 isolates from southern Italy. A total of 67 different isolates from a previous cross-sectional study conducted in the Calabria region of Italy during 2018 were selected for *de novo* WGS (6, 13). Isolates were selected according to the following criteria: (i) belonging to the predominant *spa* types circulating in EU countries, namely, t011 ($n = 45$), t034 ($n = 12$), and t899 ($n = 10$) (3, 15, 16, 25, 26); (ii) originating from farms ($n = 10$) in which LA-MRSA ST398 was isolated from both pigs and farm workers (13); and (iii) showing distinct genotypic (i.e., *spa* type or SCC*mec* type) and phenotypic (antimicrobial susceptibility pattern) traits compared with other isolates from the same farm (6, 13) (Table S1). Therefore, for each farm, isolates displaying identical epidemiological types and antimicrobial susceptibility profiles were considered duplicates, and only one representative isolate (from either pig or worker) was selected for WGS (Table S1).

DNA extraction and WGS. Genomic DNA was extracted by using the QIAamp DNA minikit (Qiagen) according to the manufacturer's instructions, except for the addition of 50 μg/ml lysostaphin (Sigma-Aldrich) during the lysis step. DNA libraries were prepared using the Nextera XT DNA library preparation kit (Illumina, San Diego, CA), and WGS was performed using a MiSeq (Illumina) platform with paired-end operating mode (2×250 bp).

Prediction of antimicrobial resistance genes. The ResFinder v.3.2 web-based pipeline at the Centre for Genomic Epidemiology (http://www.genomicepidemiology.org) was used to search for the presence of known antibiotic resistance genes using default settings (identity threshold, ≥90%; minimum length, ≥60%) (27). Genomes were screened for the *czrC* gene, encoding resistance to cadmium and zinc, by aligning sequence reads against the reference sequence (GenBank accession no. KF593809).

SNP calling and phylogenetic analysis. For comparative purposes, 89 *S. aureus* ST398 from a worldwide collection (16), plus 45 Italian t899 and t899-related ST398 isolates (15), and 283 Danish ST398 isolates (5) were included in the phylogenetic analysis. Metadata for all isolates is provided in Data Set S1 in the supplemental material.

Identification of SNPs was performed with NASP version v.1.0.0 (28) using the GATK Unified Genotyper with a filtering set to remove SNPs with less than 10-fold sequencing depth and 90% unambiguous variant calls after duplicated regions of the LA-MRSA ST398 reference chromosome S0385 (GenBank accession no. AM990992) (29) were excluded using NUCmer. SNPs caused by recombination events were identified and removed using Gubbins v.2.3.4 (30) prior to phylogenetic reconstruction using IQ-TREE version v.1.5.5 (31) with the best model found by the implemented ModelFinder and bootstrap analysis using 100 replicates. The tree was rooted according to the outgroup used in reference 16.

Statistical analysis. Data analyses were performed using GraphPad Prism v.6.1. Categorical data were compared with a two-sided Fisher's exact test. Significance was defined as a P value of ≤0.05.

Data availability. WGS data for 10 isolates from farms 01CZ and 32RC were previously submitted to the NCBI Sequence Read Archive (SRA; available at https://www.ncbi.nlm.nih.gov/sra) under BioProject PRJNA546229. WGS data for the remaining 57 isolates have been submitted to the NCBI SRA under BioProject PRJNA607440.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 4.6 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

ACKNOWLEDGMENTS

This work was supported by the BRIC ID 18/2016 grant from the Italian Workers' Compensation Authority (INAIL) to F.C., G.S., and P.V., an Excellence Departments grant from the Italian Ministry of Education, University and Research (MIUR, Italy) (Art. 1,

commi 314-337 Legge 232/2016) to the Department of Science, Roma Tre University, and the PRIN 2017 grant prot. 20177J5Y3P from MIUR to P.V. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

We report no potential conflicts of interest.

REFERENCES

1. Fluit AC. 2012. Livestock-associated *Staphylococcus aureus*. Clin Microbiol Infect 18:735–744. https://doi.org/10.1111/j.1469-0691.2012.03846.x.
2. European Food Safety Authority. 2009. Analysis of the baseline survey on the prevalence of methicillin-resistant Staphylococcus aureus (MRSA) in holdings with breeding pigs, in the EU, 2008 [1]—part A: MRSA prevalence estimates. EFSA J 7:1376. https://doi.org/10.2903/j.efsa.2009.1376.
3. Peeters LEJ, Argudín MA, Azadikhah S, Butaye P. 2015. Antimicrobial resistance and population structure of *Staphylococcus aureus* recovered from pigs farms. Vet Microbiol 180:151–156. https://doi.org/10.1016/j.vetmic.2015.08.018.
4. Grøntvedt CA, Elstrøm P, Stegger M, Skov RL, Skytt Andersen P, Larssen KW, Urdahl AM, Angen Ø, Larsen J, Åmdal S, Løtvedt SM, Sunde M, Bjørnholt JV. 2016. Methicillin-resistant *Staphylococcus aureus* CC398 in humans and pigs in Norway: a "One Health" perspective on introduction and transmission. Clin Infect Dis 63:1431–1438. https://doi.org/10.1093/cid/ciw552.
5. Sieber RN, Skov RL, Nielsen J, Schulz J, Price LB, Aarestrup FM, Larsen AR, Stegger M, Larsen J. 2018. Drivers and dynamics of methicillin-resistant livestock-associated *Staphylococcus aureus* CC398 in pigs and humans in Denmark. mBio 9:e02142-18. https://doi.org/10.1128/mBio.02142-18.
6. Pirolo M, Gioffrè A, Visaggio D, Gherardi M, Pavia G, Samele P, Ciambrone L, Di Natale R, Spatari G, Casalinuovo F, Visca P. 2019. Prevalence, molecular epidemiology, and antimicrobial resistance of methicillin-resistant *Staphylococcus aureus* from swine in southern Italy. BMC Microbiol 19:51. https://doi.org/10.1186/s12866-019-1422-x.
7. Larsen J, Petersen A, Sørum M, Stegger M, van Alphen L, Valentiner-Branth P, Knudsen LK, Larsen LS, Feingold B, Price LB, Andersen PS, Larsen AR, Skov RL. 2015. Meticillin-resistant *Staphylococcus aureus* CC398 is an increasing cause of disease in people with no livestock contact in Denmark, 1999 to 2011. Euro Surveill 20:30021. https://doi.org/10.2807/1560-7917.ES.2015.20.37.30021.
8. Sieber RN, Larsen AR, Urth TR, Iversen S, Møller CH, Skov RL, Larsen J, Stegger M. 2019. Genome investigations show host adaptation and transmission of LA-MRSA CC398 from pigs into Danish healthcare institutions. Sci Rep 9:1–10. https://doi.org/10.1038/s41598-019-55086-x.
9. Gonçalves da Silva A, Baines SL, Carter GP, Heffernan H, French NP, Ren X, Seemann T, Bulach D, Kwong J, Stinear TP, Howden BP, Williamson DA. 2017. A phylogenomic framework for assessing the global emergence and evolution of clonal complex 398 methicillin-resistant *Staphylococcus aureus*. Microb Genom 3:e000105. https://doi.org/10.1099/mgen.0.000105.
10. EUROSTAT. 2018. Agriculture, forestry and fishery statistics. https://ec.europa.eu/eurostat/documents/3217494/9455154/KS-FK-18-001-EN-N.pdf/a9ddd7db-c40c-48c9-8ed5-a8a90f4faa3f. Accessed 17 February 2020.
11. Battisti A, Franco A, Merialdi G, Hasman H, Iurescia M, Lorenzetti R, Feltrin F, Zini M, Aarestrup FM. 2010. Heterogeneity among methicillin-resistant *Staphylococcus aureus* from Italian pig finishing holdings. Vet Microbiol 142:361–366. https://doi.org/10.1016/j.vetmic.2009.10.008.
12. Parisi A, Caruso M, Normanno G, Latorre L, Miccolupo A, Fraccalvieri R, Intini F, Manginelli T, Santagada G. 2019. MRSA in swine, farmers and abattoir workers in Southern Italy. Food Microbiol 82:287–293. https://doi.org/10.1016/j.fm.2019.03.003.
13. Pirolo M, Visaggio D, Gioffrè A, Artuso I, Gherardi M, Pavia G, Samele P, Ciambrone L, Di Natale R, Spatari G, Casalinuovo F, Visca P. 2019. Unidirectional animal-to-human transmission of methicillin-resistant *Staphylococcus aureus* ST398 in pig farming; evidence from a surveillance study in southern Italy. Antimicrob Resist Infect Control 8:187. https://doi.org/10.1186/s13756-019-0650-z.
14. Espinosa-Gongora C, Broens EM, Moodley A, Nielsen JP, Guardabassi L. 2012. Transmission of MRSA CC398 strains between pig farms related by trade of animals. Vet Rec 170:564–564. https://doi.org/10.1136/vr.100704.
15. Larsen J, Stegger M, Andersen PS, Petersen A, Larsen AR, Westh H, Agersø Y, Fetsch A, Kraushaar B, Käsbohrer A, Feβler AT, Schwarz S, Cuny C, Witte W, Butaye P, Denis O, Haenni M, Madec J-Y, Jouy E, Laurent F, Battisti A, Franco A, Alba P, Mammina C, Pantosti A, Monaco M, Wagenaar JA, de Boer E, van Duijkeren E, Heck M, Domínguez L, Torres C, Zarazaga M, Price LB, Skov RL. 2016. Evidence for human adaptation and foodborne transmission of livestock-associated methicillin-resistant *Staphylococcus aureus*. Clin Infect Dis 63:1349–1352. https://doi.org/10.1093/cid/ciw532.
16. Price LB, Stegger M, Hasman H, Aziz M, Larsen J, Andersen PS, Pearson T, Waters AE, Foster JT, Schupp J, Gillece J, Driebe E, Liu CM, Springer B, Zdovc I, Battisti A, Franco A, Żmudzki J, Schwarz S, Butaye P, Jouy E, Pomba C, Porrero MC, Ruimy R, Smith TC, Robinson DA, Weese JS, Arriola CS, Yu F, Laurent F, Keim P, Skov R, Aarestrup FM. 2012. *Staphylococcus aureus* CC398: host adaptation and emergence of methicillin resistance in livestock. mBio 3:e00305-11. https://doi.org/10.1128/mBio.00305-11.
17. Simoes AJ, Hidalgo CA. 2011. The Economic Complexity Observatory: an analytical tool for understanding the dynamics of economic development. Workshops at the Twenty-Fifth AAAI Conference on Artificial Intelligence. https://static1.squarespace.com/static/5759bc7886db431d658b7d33/t/5c6e037f9140b7a9ae975475/1550713737915/SimoesHidalgo.pdf. Accessed 2 May 2020.
18. Danish Integrated Antimicrobial Resistance Monitoring and Research Programme. 2019. DANMAP 2018—use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Statens Serum Institut, National Veterinary Institute and National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark. https://www.danmap.org/-/media/arkiv/projekt-sites/danmap/danmap-reports/danmap-2018/danmap_2018.pdf. Accessed 17 February 2020.
19. EUROSTAT. 2014. Pig farming in the European Union, considerable variations from one member state to another. https://ec.europa.eu/eurostat/statistics-explained/pdfscache/3688.pdf. Accessed 17 February 2020.
20. Schulz J, Boklund A, Halasa THB, Toft N, Lentz H. 2017. Network analysis of pig movements: loyalty patterns and contact chains of different holding types in Denmark. PLoS One 12:e0179915. https://doi.org/10.1371/journal.pone.0179915.
21. Hansen JE, Ronco T, Stegger M, Sieber RN, Fertner ME, Martin HL, Farre M, Toft N, Larsen AR, Pedersen K. 2019. LA-MRSA CC398 in dairy cattle and veal calf farms indicates spillover from pig production. Front Microbiol 10:2733. https://doi.org/10.3389/fmicb.2019.02733.
22. Li H, Andersen PS, Stegger M, Sieber RN, Ingmer H, Staubrand N, Dalsgaard A, Leisner JJ. 2019. Antimicrobial resistance and virulence gene profiles of methicillin-resistant and -susceptible *Staphylococcus aureus* from food products in Denmark. Front Microbiol 10:2681. https://doi.org/10.3389/fmicb.2019.02681.
23. Argudín MA, Lauzat B, Kraushaar B, Alba P, Agerso Y, Cavaco L, Butaye P, Porrero MC, Battisti A, Tenhagen B-A, Fetsch A, Guerra B. 2016. Heavy metal and disinfectant resistance genes among livestock-associated methicillin-resistant *Staphylococcus aureus* isolates. Vet Microbiol 191:88–95. https://doi.org/10.1016/j.vetmic.2016.06.004.
24. Moodley A, Nielsen SS, Guardabassi L. 2011. Effects of tetracycline and zinc on selection of methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type 398 in pigs. Vet Microbiol 152:420–423. https://doi.org/10.1016/j.vetmic.2011.05.025.
25. Guardabassi L, O'Donoghue M, Moodley A, Ho J, Boost M. 2009. Novel lineage of methicillin-resistant *Staphylococcus aureus*, Hong Kong. Emerg Infect Dis 15:1998–2000. https://doi.org/10.3201/eid1512.090378.
26. Dierikx CM, Hengeveld PD, Veldman KT, de Haan A, van der Voorde S, Dop PY, Bosch T, van Duijkeren E. 2016. Ten years later: still a high

prevalence of MRSA in slaughter pigs despite a significant reduction in antimicrobial usage in pigs the Netherlands. J Antimicrob Chemother 71:2414–2418. https://doi.org/10.1093/jac/dkw190.
27. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67:2640–2644. https://doi.org/10.1093/jac/dks261.
28. Sahl JW, Lemmer D, Travis J, Schupp JM, Gillece JD, Aziz M, Driebe EM, Drees KP, Hicks ND, Williamson CHD, Hepp CM, Smith DE, Roe C, Engelthaler DM, Wagner DM, Keim P. 2016. NASP: an accurate, rapid method for the identification of SNPs in WGS datasets that supports flexible input and output formats. Microb Genom 2:e000074. https://doi.org/10.1099/mgen.0.000074.
29. Schijffelen MJ, Boel CE, van Strijp JA, Fluit AC. 2010. Whole genome analysis of a livestock-associated methicillin-resistant *Staphylococcus aureus* ST398 isolate from a case of human endocarditis. BMC Genomics 11:376. https://doi.org/10.1186/1471-2164-11-376.
30. Croucher NJ, Page AJ, Connor TR, Delaney AJ, Keane JA, Bentley SD, Parkhill J, Harris SR. 2015. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic Acids Res 43:e15. https://doi.org/10.1093/nar/gku1196.
31. Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol 32:268–274. https://doi.org/10.1093/molbev/msu300.